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The role of TGF-β signaling in regulating chondrogenesis and osteogenesis during mandibular development

Kyoko Oka [a], Shoji Oka [a], Tomoyo Sasaki [a], Yoshihiro Ito [a], Pablo Bringas Jr. [a], Kazuaki Nonaka [b], Yang Chai [a,*]

[a] Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, 2250 Alcazar Street, CSA 103, Los Angeles, CA 90033, USA
[b] Section of Pediatric Dentistry, Division of Oral Health, Growth and Development, Kyushu University, Maidashi 3-1-1, Higashi-Ku, Fukuoka 812-8582, Japan

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Abstract

During craniofacial development, Meckel's cartilage and the mandible bone derive from the first branchial arch, and their development depends upon the contribution of cranial neural crest (CNC) cells. We previously demonstrated that conditional inactivation of *Tgfbr2* in the neural crest of mice (*Tgfbr2*$^{fl/fl}$*;Wnt1-Cre*) results in severe defects in mandibular development, although the specific cellular and molecular mechanisms by which TGF-β signaling regulates the fate of CNC cells during mandibular development remain unknown. We show here that loss of *Tgfbr2* does not affect the migration of CNC cells during mandibular development. TGF-β signaling is specifically required for cell proliferation in Meckel's cartilage and the mandibular anlagen and for the formation of the coronoid, condyle and angular processes. TGF-β-mediated connective tissue growth factor (CTGF) signaling is critical for CNC cell proliferation. Exogenous CTGF rescues the cell proliferation defect in Meckel's cartilage of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutants, demonstrating the biological significance of this signaling cascade in chondrogenesis during mandibular development. Furthermore, TGF-β signaling controls *Msx1* expression to regulate mandibular osteogenesis as *Msx1* expression is significantly reduced in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutants. Collectively, our data suggest that there are differential signal cascades in response to TGF-β to control chondrogenesis and osteogenesis during mandibular development.

Keywords: Cranial neural crest (CNC); Cell proliferation; Differentiation; Mandible and Meckel's cartilage development; CTGF; *Msx1*; TGF-β

Introduction

The mandible is an important structure involved in the essential functions of breathing and mastication. Many human syndromes with craniofacial anomalies include defects in mandible formation. Mandibular development depends on the processes of chondrogenesis and osteogenesis. Specifically, chondrogenesis involves the formation of Meckel's cartilage and coronoid, condylar and angular cartilage. Mandible bone is formed by intramembranous ossification. To date, little is known about the regulation of chondrogenesis and osteogenesis during mandibular development.

Meckel's cartilage appears transiently during the early embryonic stage. Previous studies have suggested that the role of Meckel's cartilage is to serve as a template for mandible formation and also to contribute as part of the mandible bone after ossification (Carda et al., 2005; Melnick et al., 2005; Ramaesh and Bard, 2003). Meckel's cartilage, derived from the first branchial arch, arises from an ectomesenchymal cell population of cranial neural crest cells (Chai et al., 1998; Hall, 1980). It contains three distinct regions, each having a different fate. The distal region of Meckel's cartilage contributes to mandibular development and undergoes endochondral-like ossification. This region fuses with the rostrum during an early developmental stage and, subsequently, the medial part of the rostrum gives rise to the symphysis region of the mandible. The middle region, the cartilage distal to the ossification center of the mandibular angle, gives rise to the sphenomandibular ligament. Finally, the most proximal region of Meckel's cartilage, which gives rise to the malleus and incus, also undergoes endochondral ossification (Frommer and Margolies, 1971; Savostin-Asling and Asling, 1973).

* Corresponding author. Fax: +1 323 442 2981.
E-mail address: ychai@usc.edu (Y. Chai).

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The coronoid, condylar and angular cartilage are classified as secondary cartilage (Beresford, 1975). The early mandible bone is formed by intramembranous ossification, but like the epiphyseal cartilage, its proximal end contributes to endochondral components at later stages. The condylar cartilage also serves as an important growth center in the proximal part of the developing mandible (Lee et al., 2001).

Members of the transforming growth factor-β (TGF-β) superfamily mediate a wide range of biological activities, including cell proliferation, differentiation and extracellular matrix formation, suggesting that TGF-β signaling is important during embryogenesis (Chai et al., 2003). TGF-β signaling stimulates proliferation and inhibits terminal differentiation of chondrocytes during chondrogenesis (Alvarez et al., 2001; Serra et al., 1997). We have previously reported the dynamic distribution of cranial neural crest cells (CNC) during Meckel's cartilage development and the biological function of TGF-β signaling in regulating the proliferation of CNC-derived chondrocytes (Ito et al., 2002). Mice with *Tgfbr2* conditional gene ablation in the CNC (*Tgfbr2$^{fl/fl}$;Wnt1-Cre*) have craniofacial anomalies including defects in mandible development (Ito et al., 2003). Specifically, these mice have small mandibles, diminished coronoid and condylar processes and missing angular processes. Despite these observations, the downstream mediators of TGF-β signaling to regulate mandible development have remained unclear.

Connective tissue growth factor (CTGF, CCN2) is a member of the CCN family of secreted proteins. CTGF is involved in a variety of developmental processes, including cell determination and differentiation, morphogenesis, and cell migration and proliferation. CTGF is believed to be a downstream mediator of TGF-β action (Chaqour and Goppelt-Struebe, 2006; Grotendorst, 1997) because TGF-β induces the expression of CTGF in fibroblasts (Kikuchi et al., 1995) and chondrocytes (Nakanishi et al., 1997; Shimo et al., 2004) and the CTGF promoter sequence contains a TGF-β response element (Grotendorst et al., 1996; Takigawa et al., 2003). Recently, a study has shown that *Ctgf* mutant mice exhibit deformation of Meckel's cartilage and shortened mandibles (Ivkovic et al., 2003). These mandible phenotypes are quite similar to those of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice.

Here we examine *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice in order to elucidate the role of TGF-β and CTGF in mandible development. We find that the cell proliferation of chondrocytes in Meckel's cartilage and of the oseteogenic front of the mandibular bone is reduced. *Ctgf* mRNA expression is decreased in the perichondrium of Meckel's cartilage. Strikingly, exogenous CTGF rescues the cell proliferation defect in Meckel's cartilage of the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant. *Msx1* expression is also reduced in the mandibular primordium of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. Finally, the condylar process in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice has lost the zonation typical of endochondral ossification. This defect begins during the initiation stage of condylar development. Moreover, there are no chondrocytes in the angular process of mandible in the *Tgfbr2* mutant. Collectively, our study provides evidence for the functional significance of TGF-β signaling during mandibular development. *Tgfbr2$^{fl/fl}$;Wnt1-Cre* conditional knockout mice can serve as a useful animal model that will facilitate a comprehensive understanding of normal craniofacial development as well as CNC-related congenital malformations.

Materials and methods

Generation of Wnt1-Cre;R26R and Tgfbr2$^{fl/fl}$;Wnt1-Cre mutant mice and histological analysis

The *Wnt1-Cre* transgenic line and *R26R* conditional reporter allele have been described previously (Danielian et al., 1998; Soriano, 1999). We crossed *Wnt1-Cre* and *R26R* mice to produce genetically labeled neural crest cells that could be identified with β-galactosidase (Chai et al., 2000). Mating *Tgfbr2$^{fl/+}$;Wnt1-Cre* with *Tgfbr2$^{fl/fl}$* mice generated *Tgfbr2$^{fl/fl}$;Wnt1-Cre* null allele progeny that were genotyped using PCR primers as previously described (Chytil et al., 2002). Samples with no detected Cre transgene were used as controls. All samples were fixed in 4% paraformaldehyde and processed into serial paraffin sections using routine procedures. For general morphology, deparaffinized sections were stained with Hematoxylin and Eosin or Safranin-O staining.

Cryostat sectioning for X-gal staining

Mouse embryonic tissue was frozen, sectioned and then stained according to standard procedures. Specifically, mouse tissue was dissected in PBS and fixed by immersion in 0.2% glutaraldehyde solution overnight at 4 °C. Tissue was soaked in 10% sucrose in PBS for 30 min at 4 °C, incubated in PBS plus 2 mM $MgCl_2$, 30% sucrose and frozen in OCT. Sections were cut at 8–12 μm and mounted on polylysine-coated slides. The mounted tissue sections were post-fixed in 0.2% glutaraldehyde for 10 min on ice, rinsed briefly in PBS and rinsed in detergent solution (0.005% NP-40 and 0.01% sodium deoxycholate in PBS) for 10 min at 4 °C. Thereafter, the slides were washed in PBS for 10 min and stained in X-gal staining solution overnight at 37 °C in the dark. Sections were counterstained with Nuclear Fast Red.

Analysis of cell proliferation and apoptosis

DNA synthesis activity within Meckel's cartilage and mandibular bone was monitored by intraperitoneal BrdU (5-bromo-2′-deoxy-uridine, Sigma) injection (100 μg/g body weight) at E11.5, 12.5 and 13.5. One hour after injection, mice were sacrificed and embryos were fixed in 10% buffered formalin solution and processed. Serial sections of the specimen were cut at 7 μm intervals. In each embryonic sample, three randomly selected sections from the middle region of mandible were used for cell proliferation or apoptosis analysis. We detected BrdU labeled cells using a BrdU Labeling and Detection Kit by following manufacturer's protocol (Zymed). We scored the BrdU-positive and the total number of cells within the Meckel's cartilage and mandible bone. Student's *t*-test was applied for statistical analysis. A p value of less than 0.05 was considered statistically significant. TUNEL assay was performed using the In Situ Cell Death Detection (fluorescein) Kit (Roche Molecular Biochemicals) by following the manufacturer's protocol.

In situ hybridization

We performed in situ hybridizations following standard procedures. PFA (4%)-fixed samples were dehydrated by passage through a graded ethanol series. The dehydrated samples were subsequently embedded in paraffin in preparation for non-radioactive and radioactive in situ hybridization. Serial tissue sections were treated with proteinase K for 15 min at room temperature. All riboprobes were generated by in vitro transcription using ^{33}P-UTP-labeled or digoxigenin-UTP-labeled and according to the manufacturer's instructions (Roche Diagnostics Corp.). Several negative controls (e.g. sense probe and no probe) were run in parallel with the experimental reaction.

Organ culture of wild type and Tgfbr2$^{fl/fl}$;Wnt1-Cre mutant mandible bone primordium explants

Timed-pregnant mice were sacrificed on post-coital day 12.5 and staged according to external developmental characteristics. BSA and TGF-β2 or CTGF beads were implanted in lateral side of Meckel's cartilage. Mandible explants (six per treatment group) were cultured for 24 h in serumless, chemically defined medium according to standard methods (Ito et al., 2002).

Preparation and introduction of TGF-β or CTGF beads

We used affi-gel blue beads (Bio-Rad) for delivery of TGF-β2. The beads were washed in phosphate-buffered PBS and then incubated for 1 h at room temperature in 10 μg/ml TGF-β2 (R&D). Heparin-acrylic beads (Sigma) were incubated in CTGF (20 ng/ml, cell sciences) for 1 h. Control beads were incubated in 0.1% BSA. In both control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples, one side of the mandible was treated with BSA beads and the other side treated with TGF-β2 or CTGF beads.

Real-time quantitative RT-PCR

The mRNA levels of *Msx1*, *Msx2* and *Ctgf* were analyzed by real-time quantitative RT-PCR (Bio-Rad iCycler system). Meckel's cartilage was dissected at E13.5 and total RNA was extracted. The mRNAs were reverse-transcribed into cDNAs by using SuperScript™ First-Strand (Invitrogen life technologies). The real-time PCR was performed using a SYBR super mix kit (Bio-Rad), running for 40 cycles at 95 °C for 15 s and 60 °C for 45 s. The melting curve data were collected to check the PCR specificity. Each cDNA sample was analyzed in triplicate. The threshold cycle (CT) was defined as the fractional cycle number. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values; ΔCt=Ctinterest−CtGapdh) between the genes of interest (*Msx1*, *Msx2* and *Ctgf*) and an internal reference gene (*Gapdh*) that provides a normalization factor for the amount of RNA isolated from a specimen. By using the Δ(ΔCt) method, {Δ(ΔCt)=ΔCtmutant−ΔCtcontrol}, the fold change $\{2^{-\Delta(\Delta Ct)}\}$ was calculated for each control and mutant samples. All data are shown as mean±SD, Student's *t*-test was applied for statistical analysis. A p value of less than 0.05 was considered statistically significant.

Results

TGF-β signaling is required for proper development of Meckel's cartilage and the mandibular bone

As *Wnt1* transgene expression is limited to migrating neural crest cells, we generated mice with conditional inactivation of *Tgfbr2* gene (*Wnt1-Cre;Tgfbr2$^{fl/fl}$*) in all neural crest derived cells (Ito et al., 2003). All *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice had identical mandible defects, consisting of small mandible bones, diminished condylar and coronoid processes and absent angular processes (Figs. 1A–F). Even as the mandible bone was forming on the side of Meckel's cartilage at E14.5, its size was already smaller in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples than that of control (Figs. 1G, H). Furthermore, we observed an abnormal shape of Meckel's cartilage at this stage, with a curvy form and non-uniform thickness areas (Figs. 1F, H). We performed histological analyses of Meckel's cartilage in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutants at multiple time points in order to characterize this defect. As early as E13.5, we detected a deformed shape of Meckel's cartilage in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice (Figs. 1K, L). In addition, the visible layers of the perichondrium in Meckel's cartilage were disrupted (Figs. 1M, N arrow). At earlier stages, the Meckel's cartilage in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant sample was indistinguishable from the control samples (Figs. 1I, J).

Loss of TGF-β signaling does not affect the migration of CNC cells into Meckel's cartilage and the mandible primordium

In order to test whether a CNC migration defect might contribute to the deficiency of the Meckel's cartilage and the mandible bone primordium, we generated *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mice. All of these embryos had identical malformations to those seen in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice. At E10.5, the first branchial arch had divided into maxillary and mandibular prominences and *lacZ* expression was detectable in the frontonasal, maxillary and mandibular prominences surrounding the olfactory pit (data not shown). Whole-mount staining has previously shown no difference in migration and distribution of CNC cells within the first branchial arch of *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutant and control embryos from E9.5 to E11.5 (Ito et al., 2003). After sectioning and staining samples at E12.5, we found that the perichondrium and the chondrocytes of Meckel's cartilage were primarily composed of CNC-derived cells. The mandibular bone primordium began to form on the lateral side of Meckel's cartilage (Figs. 2A, B). At E14.5, the mandible primordium had formed bone matrix and the size of the mandible bone in *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mice was smaller than that of *R26R;Wnt1-Cre* mice. We also observed that the perichondrium of the Meckel's cartilage was disorganized. However, the contribution of CNC-derived cells in *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* was comparable to control (Figs. 2C, D). In addition, we verified the deletion of *Tgfbr2* gene in CNC-derived Meckel's cartilage and mandible. In situ analysis showed that *Tgfbr2* was expressed in Meckel's cartilage and mandible bone primordium in the control sample while it was not detectable in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant (Figs. 2E, F). Taken together, our data suggest that there was no CNC migration defect in the forming Meckel's cartilage and mandible in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice.

Loss of TGF-β signaling perturbs proliferation but not survival of CNC cells

We investigated whether there was a decrease in cell proliferation in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples by comparing BrdU incorporation in the chondrocytes of Meckel's cartilage and the osteogenic front of the mandible bone primordium with the control mice (Figs. 3A–F). At E11.5, we did not observe any defects in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutants by histological analysis. There was no apparent reduction in cell proliferation activity of chondrocytes in Meckel's cartilage in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant when compared with the control (control, $n=6$; mutant, $n=6$; Figs. 3A, B). At E12.5, Meckel's cartilage in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant still did not show any abnormality in form, but we found a significant reduction in cell proliferation activity

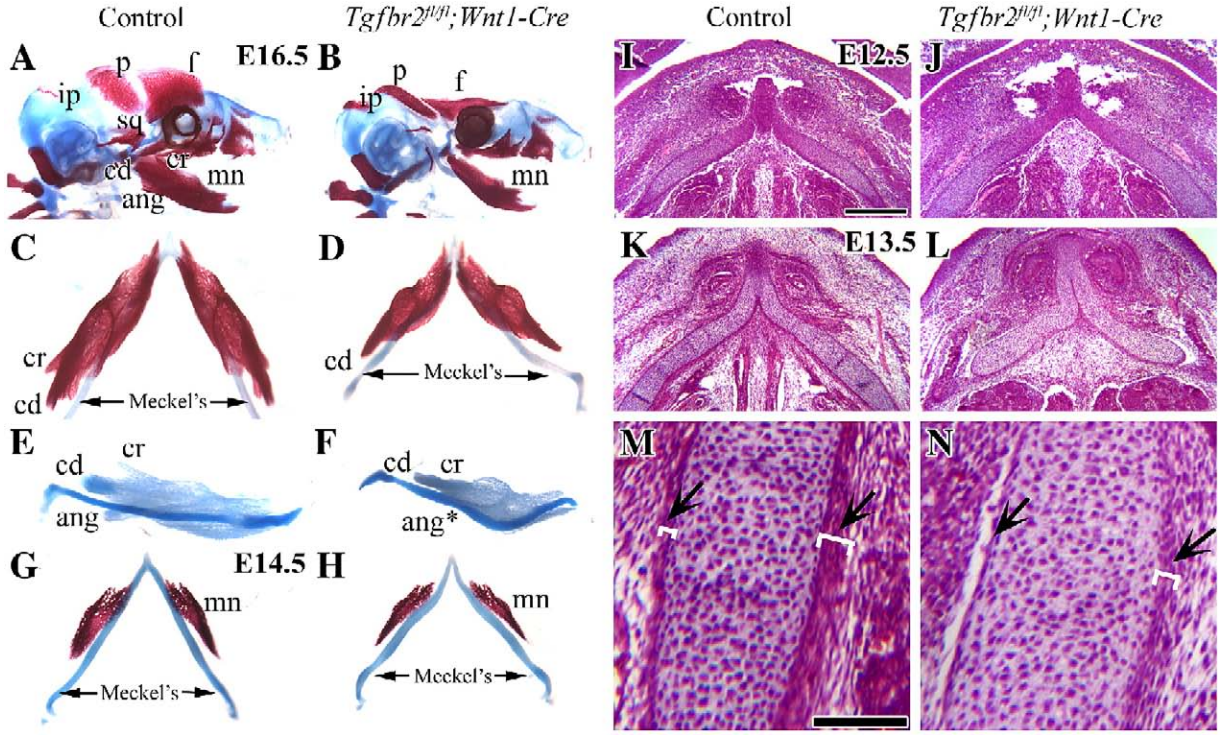


Fig. 1. Developmental defects during the formation of Meckel's cartilage and the mandible bone in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice. (A, B) Lateral view of skeletal staining preparations of control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice at E16.5. *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice have severe defects of the frontal (f), parietal (p) and mandible bones (mn). (C, D) Top view of Meckel's cartilage and the mandible bone at E16.5 in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice. (E, F) Lateral view of mandible complexes stained with Alcian Blue at E16.5 in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice. *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice have a shortened mandible bone, diminished coronoid and condylar process and absent angular process. (G, H) Top view of Meckel's cartilage and the mandible bone at E14.5 in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice samples. (I–M) Histological analysis of Meckel's cartilage in transversal sections at E12.5 (I, J) and E13.5 (K–N). At E12.5, the Meckel's cartilage in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant sample is indistinguishable from control. At E13.5, the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant sample has defects including a curved Meckel's cartilage and disrupted layers of the perichondrium and alignment of the chondrocytes in Meckel's cartilage (arrows). The white parentheses indicate the thickness of the layers. Scale bars: 200 μm in panels I–L; 100 μm in panels M, N. f; frontal bone, p; parietal bone, ip; interparietal bone, sq; squamous bone, mn; mandible bone, cd; condylar process, cr; coronoid process, ang; angular process, Meckel's; Meckel's cartilage.

($p<0.05$) within the chondrocytes of Meckel's cartilage (control, $n=6$, BrdU index = 14.9 ± 0.8%; mutant, $n=6$, BrdU index = 9.1 ± 1.2%; Figs. 3C, D, I). In the mandible primordium, cell proliferation was comparable in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant and control at E12.5. However, at E13.5, cell proliferation activity was significantly reduced in the mandibular primordium of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant within both the oral and aboral areas, as compared to control samples (control, $n=15$; mutant, $n=13$; Figs. 3E, F). Thus, the reduction in cell proliferation activity occurred before the appearance of the morphological defect in both Meckel's cartilage and the mandible primordium.

To determine whether an increase in apoptosis might have contributed to the defect in mandible development in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice, we performed TUNEL assays. At E13.5, apoptotic activity was indistinguishable in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant and the control samples (Figs. 3G, H), suggesting that loss of TGF-β signaling did not affect the survival of the CNC-derived mesenchymal cells during mandibular development. Instead, decreased cell proliferation was likely responsible for the defects of Meckel's cartilage and mandible in the absence of TGF-β signaling.

Downstream mediators of TGF-β signaling in Meckel's cartilage and mandible bone

To elucidate the molecular signaling cascade involved in regulating cell proliferation of Meckel's cartilage and mandible bone, we examined the expression of *Msx1* and *Msx2*, genes known to be crucial in regulating mandibular development. At E13.5, *Msx1* was expressed around the perichondrium of Meckel's cartilage and the osteogenic front of the mandible bone and dental mesenchyme in the control (Fig. 4A). In the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice, *Msx1* expression was lost around the perichondrium of Meckel's cartilage, decreased in the osteogenic front of mandible bone and unchanged in the dental mesenchyme (Fig. 4B). At E14.5, *Msx1* expression in control spread to mesenchymal cells between Meckel's cartilage and mandible bone. However, *Msx1* expression was reduced in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice (Figs. 4C, D). *Msx2* was expressed in the perichondrium of Meckel's cartilage and the mandible bone primordium in control mice at E13.5 and E14.5. The expression of *Msx2* was slightly decreased in the perichondrium of Meckel's cartilage in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice, but was indistinguishable

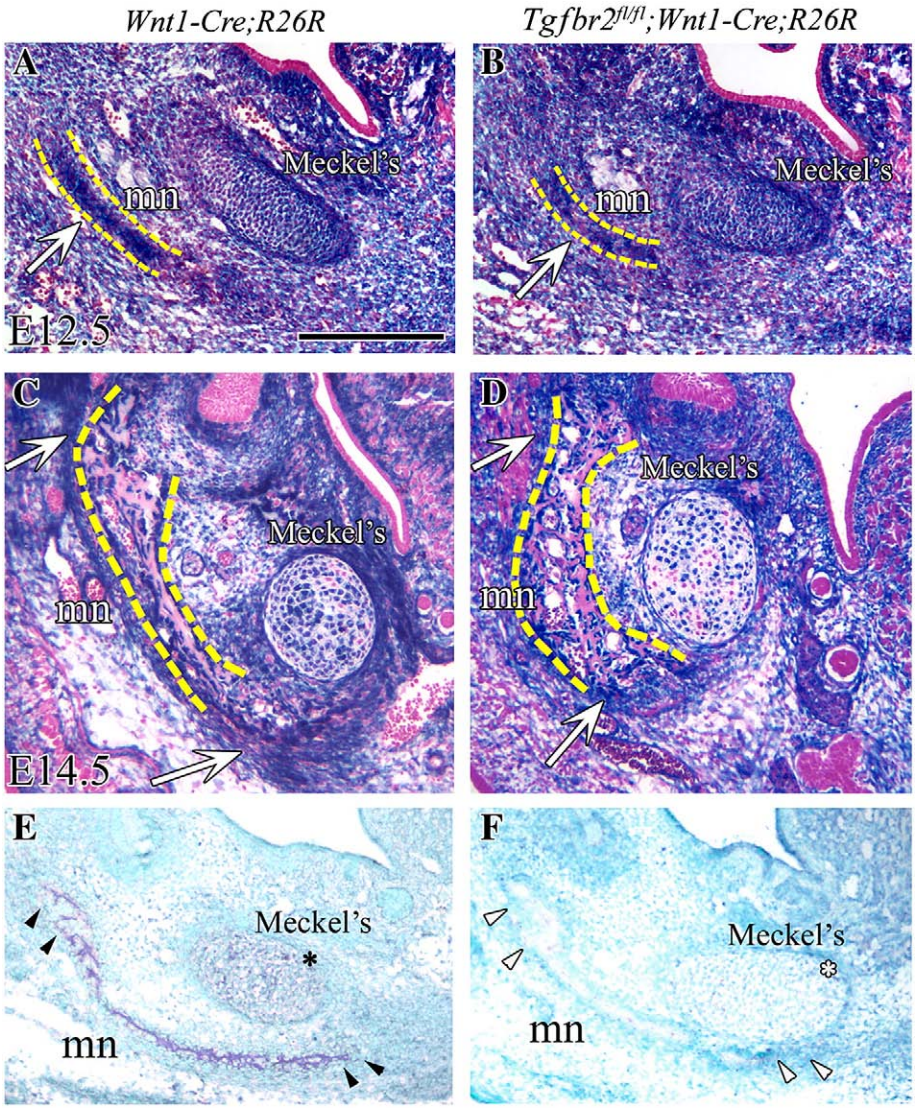


Fig. 2. CNC cell migration into Meckel's cartilage is not affected by loss of *Tgfbr2*. CNC cells are (blue) visualized by X-gal staining of the mandible at E12.5 (A, B) and E14.5 (C, D) in *R26R;Wnt1-Cre* (control) and *Tgfbr2*$^{fl/fl}$*;R26R;Wnt1-Cre* mice. (A, B) At E12.5, CNC-derived cells are detected in Meckel's cartilage and the mandible primordium (arrow). There is no apparent difference in the population of cells in Meckel's cartilage and mandible bone of *R26R;Wnt1-Cre* and *Tgfbr2*$^{fl/fl}$*;R26R;Wnt1-Cre* mice. (C, D) At E14.5, the Meckel's cartilage is composed of CNC-derived and non-CNC-derived cells. The staining reveals the high density of CNC-derived cells in the osteogenic front of the mandible primordium (arrow). Yellow dashed lines indicate the mandible bone primordium (A, B) or the mandible bone (C, D). (E, F) In situ hybridization shows that *Tgfbr2* is expressed (dark blue) in Meckel's cartilage (*) and in the mandibular primordium (arrowhead) in the control (E) while it is not detectable in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* sample (F) at E13.5. Scale bar: 200 μm in panels A–D. mn; mandible, Meckel's; Meckel's cartilage.

from control in the mandible bone (data not shown). We performed a quantitative analysis of *Msx1* and *Msx2* expression using dissected Meckel's cartilage at E13.5 and found that *Msx1*, but not *Msx2*, expression was significantly decreased ($p<0.01$) in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice (Fig. 4E and data not shown).

TGF-β signaling and osteoprogenitor differentiation during mandible development

We next analyzed the expression of osteogenic markers to determine if a disruption in osteogenic differentiation might be responsible for the defect in mandible bone development in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant. Runx2 is the earliest identified factor required for osteoblast differentiation, and Twist is a negative regulator of osteoblast differentiation that inhibits Runx2 function (Bialek et al., 2004; Jabs, 2001; Komori et al., 1997; Otto et al., 1997). *Runx2* was expressed strongly in the osteogenic front of the mandible bone (Figs. 5A, B). Interestingly, *Twist* was expressed distinctly on the buccal side of the mandibular bone primordium, in contrast to *Runx2* expression (Figs. 5C, D). *Runx2* and *Twist* expression in the developing mandible bone primordium was indistinguishable between control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant samples. Furthermore, we observed no difference in *type I collagen* (*Coll*), *osterix* (*Osx*), *Bsp* and *osteonectin* (*ON*) expression (Figs. 5E–L). These

data suggest that osteoblast differentiation and bone matrix formation appear to be normal in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice.

CTGF functions as a downstream mediator of TGF-β signaling to control cell proliferation activity within Meckel's cartilage

To test whether CTGF is a downstream target of TGF-β signaling during Meckel's cartilage development, we compared the expression of *Ctgf* in control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples. At E12.5 and E13.5, *Ctgf* mRNA was specifically expressed in the perichondrium of Meckel's cartilage in the control, but was reduced in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant (Figs. 6A–D). Next, we performed a quantitative analysis of *Ctgf* mRNA expression in Meckel's cartilage at E13.5 using real-time PCR to eliminate the possibility that the reduction of *Ctgf* expression was due to the disorganization of perichondrium. In fact, *Ctgf* expression was significantly decreased in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples ($p<0.01$) (Fig. 6E), consistent with the proposal that *Ctgf* is a downstream target of TGF-β signaling in Meckel's cartilage.

Differentiation of chondrocytes in Meckel's cartilage was accelerated in Tgfbr2$^{fl/fl}$;Wnt1-Cre mice

Ihh is a differentiation marker for maturing chondrocytes as it is expressed in differentiating chondrocytes and not expressed in proliferating or terminally differentiated chondrocytes. We examined the expression of *Ihh* in both the control and *Tgfbr2* mutant samples. In control samples, *Ihh* was expressed in the middle region of Meckel's cartilage (Fig. 7A). However, the area of *Ihh* expression was expanded in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice and included the distal part of Meckel's cartilage (Fig. 7B). This result suggests that the differentiation of chondrocytes in Meckel's cartilage was accelerated in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* samples. In fact, we did observe abnormal ossification within the Meckel's cartilage of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at birth. In the control sample, Meckel's cartilage provided the connection between the proximal part of mandible and the middle ear bones (Fig. 7C). In the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant sample, there was bone formation around the most proximal part of Meckel's cartilage that connected to the malleus and incus (Fig. 7D). These data support the conclusion that differentiation of chondrocyte in Meckel's cartilage was accelerated in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples.

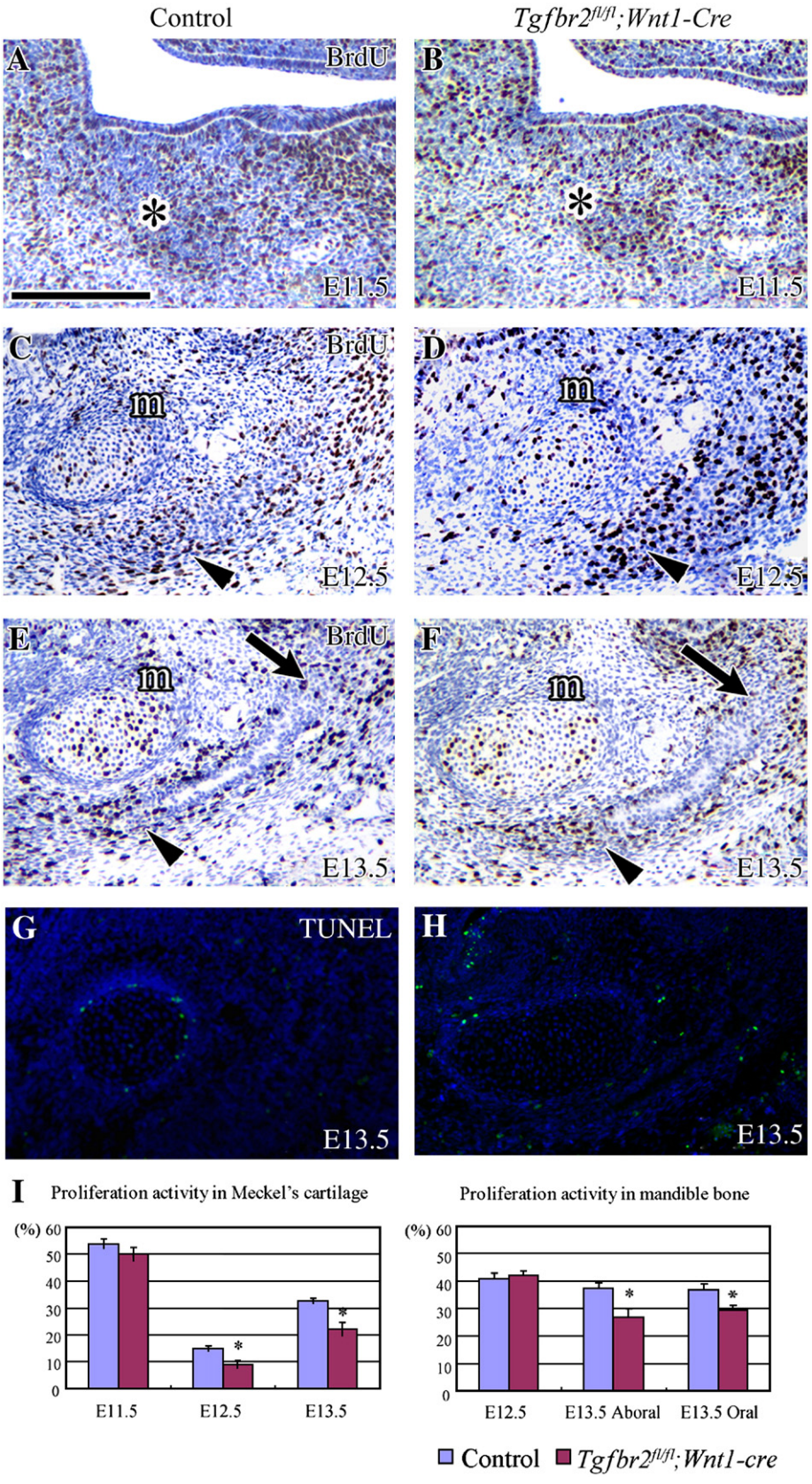
Exogenous CTGF protein rescues the cell proliferation defect in Tgfbr2$^{fl/fl}$;Wnt1-Cre mutants

We hypothesized that TGF-β-mediated CTGF signaling is critical for proliferation of chondrocytes in Meckel's cartilage. To examine this further, we treated mandible explants from *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples with TGF-β2, CTGF or BSA beads and evaluated CNC cell proliferation activity. As expected, TGF-β2 beads were able to increase cell proliferation activity within Meckel's cartilage in the control samples, and BSA beads did not (Figs. 8A, C, D). Strikingly, CTGF beads strongly stimulated cell proliferation in Meckel's cartilage and were able to rescue the cell proliferation defect in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples (cell proliferation indices: in the control sample: BSA, 29.25±4.2; in *Tgfbr2* mutant sample: BSA, 9.7±2.2; CTGF, 27.3±1.5; $p<0.01$) (Figs. 8B, E, F), while TGF-β2 failed to rescue the cell proliferation defect in the *Tgfbr2* mutant (data not shown). These results strongly suggest that CTGF is a crucial downstream component of the TGF-β signaling cascade regulating CNC cell proliferation during Meckel's cartilage development (Fig. 8G).

TGF-β signaling is required for proper development of the condylar process

In *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice, the condylar and coronoid processes of the mandible bone were defective at E16.5 (Figs. 1A–F). The distribution of CNC-derived cells was widespread in the condylar and angular cartilage, including in the regions of endochondral ossification (Fig. 9A, insets). Histological analysis showed that the zonation of endochondral ossification was similar in the condylar and angular processes in the control at E18.5 (Fig. 9A). In contrast, the size of the condylar process in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant was smaller than the one in the control samples and the zonation of endochondral ossification was lost (Fig. 9B). At E16.5, we observed well-differentiated chondrocytes and clear zonation, including articular, intermediate and hypertrophic zones, in condylar processes of control samples (Fig. 9C). In *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice, the chondrogenesis of the condylar processes was diminished and the hypertrophic zone was not present (Fig. 9D, arrow). Prior to cartilage matrix formation, we did observe the initiation stage of the condylar process as mesenchymal cell condensation in the posterior of the ossifying mandible in both control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples (data not shown). At E14.5, the condylar cartilage

Fig. 3. Cell proliferation and apoptosis during Meckel's cartilage and mandible bone development. (A–F) BrdU incorporation in the chondrocytes of Meckel's cartilage and the osteogenic front of the mandible bone primordium in control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at E11.5, E12.5 and E13.5. BrdU staining (brown spot) indicates cell proliferation activity. (A, B) At E11.5, Meckel's cartilage is visible as the highly condensed cell area (asterisk) in both control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples. (C, D) At E12.5, the Meckel's cartilage (m) is well defined and the mandible bone primordium is becoming visible as the highly condensed cell area next to Meckel's cartilage (arrowhead). (E, F) At E13.5, active CNC cell proliferation is seen in the oral (arrow) and aboral (arrowhead) side of the osteogenic front of the mandible bone, but it is significantly reduced in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant within both the oral and aboral areas. There is also a reduction of proliferation activity in Meckel's cartilage (m). (G, H) TUNEL assay in the chondrocytes of Meckel's cartilage and the mandible bone in control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at E13.5. Neither control nor *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice have detectable apoptotic activity. (I) Quantification of cell proliferation activity from panels A to F. Asterisks indicate statistical significance ($p<0.05$). Scale bar: 200 μm in panels A–H.


Control
Tgfbr2fl/fl;Wnt1-Cre
A
BrdU
E11.5
B
BrdU
E11.5
C
BrdU
m
E12.5
D
m
E12.5
E
BrdU
m
E13.5
F
m
E13.5
G
TUNEL
E13.5
H
E13.5
I
Proliferation activity in Meckel's cartilage
(%) 60
50
40
30
20
10
0
E11.5
E12.5
E13.5
Proliferation activity in mandible bone
(%) 60
50
40
30
20
10
0
E12.5
E13.5 Aboral
E13.5 Oral
Control
Tgfbr2fl/fl;Wnt1-cre

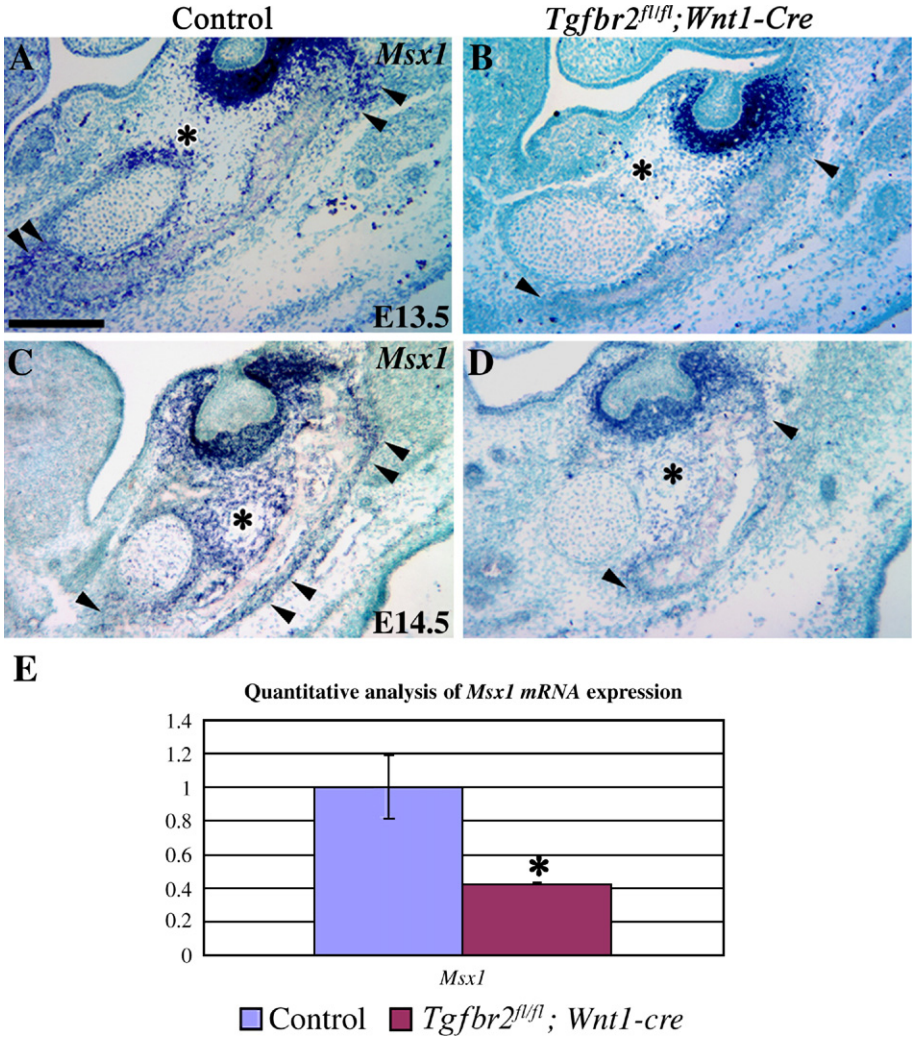


Fig. 4. *Msx1* expression is down-regulated in the Meckel's cartilage and mandible bone of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice. (A–D) In situ hybridization of *Msx1* mRNA within Meckel's cartilage and the osteogenic front of the mandible bone (arrowhead) in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice at E13.5 and E14.5. (*) Indicates *Msx1* expression in panels A and C and no *Msx1* expression in panels B and D. (E) Quantitative analysis of *Msx1* mRNA expression by real-time quantitative RT-PCR in Meckel's cartilage at E13.5. Asterisks indicate statistical significance ($p<0.01$). Scale bar: 200 μm in panels A–D.

matrix was clearly visible in wild type but not in *Tgfbr2*$^{fl/fl}$*; Wnt1-Cre* mice (Figs. 9E, F). These data suggest that the condylar process defect in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice was the result of defects in chondrocyte cell fate determination in the proximal part of the mandible.

Discussion

The mandible bone is formed by intramembranous ossification. Meckel's cartilage appears to be important in this process as a scaffold and guide for normal mandible development. Meckel's cartilage development starts as an aggregation of cranial neural crest derived mesenchymal cells at the molar tooth bud region (Ito et al., 2002). Following initiation, Meckel's cartilage extends towards the anterior and the posterior to develop a "wishbone-like" structure, with CNC-derived cells at the chondrogenic front. Although both CNC- and non-CNC-derived cells contribute to the formation of Meckel's cartilage, it is clear that CNC-derived cells play an essential role in the development of Meckel's cartilage and in the guidance of mandible bone formation (Chai et al., 2000; Ito et al., 2002).

In this study, conditional inactivation of *Tgfbr2* in neural crest cells results in abnormal formation of Meckel's cartilage and affects the morphology of mandible development. Of the three mammalian TGF-β isoforms, TGF-β1, -β2 and -β3, only the *Tgfb2* knockout mutant has a mandible defect. The mandible does not form a wishbone structure in the *Tgfb2* mutant and the coronoid and condylar processes are diminished to approximately one-half their normal dimensions (Sanford et al., 1997). Similarly, conditional inactivation of *Alk5* (TGF-β type I receptor) in neural crest cells results in malformation of Meckel's cartilage and the mandible (Dudas et al., 2006). Furthermore, previous studies have suggested that malformation of Meckel's cartilage may adversely affect the development of mandible bone. For example, *Ctgf*$^{-/-}$ mutant mice have an abnormal formation of Meckel's cartilage and a shortened mandible bone (Ivkovic et al., 2003). Conditional inactivation

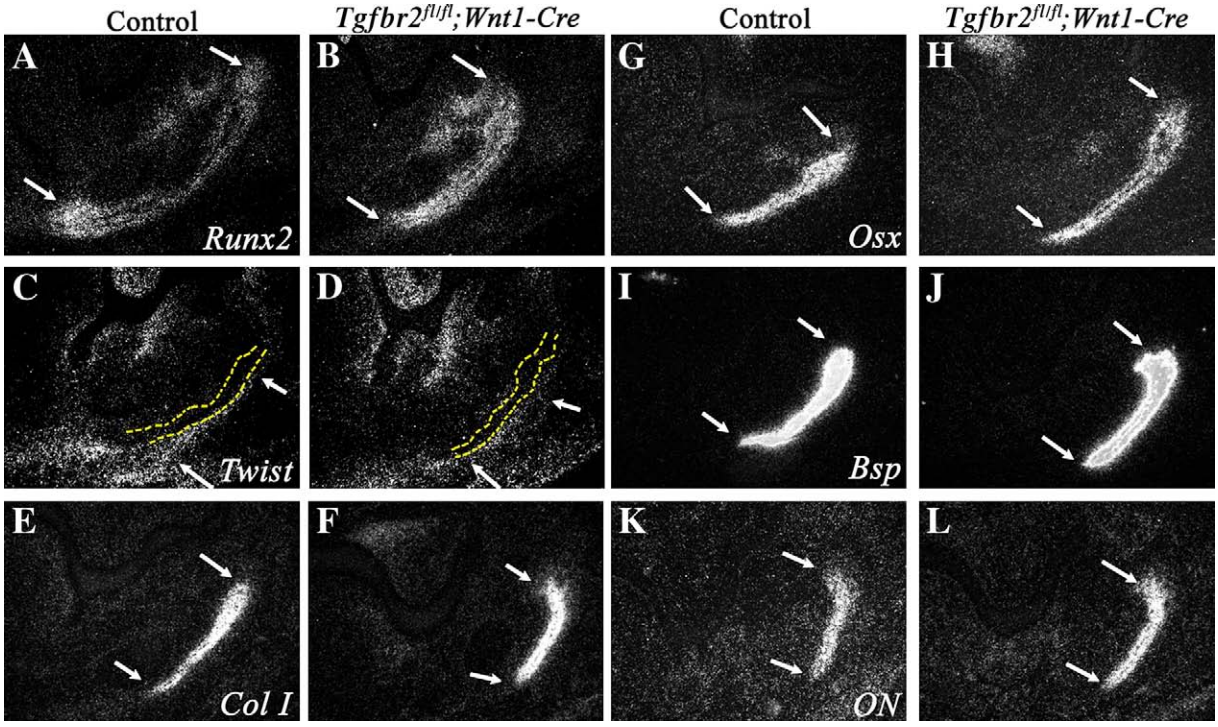


Fig. 5. TGF-β signaling is not required for bone matrix maturation in the developing mandible bone. In situ hybridization of the mandible bone primordium at E13.5 in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice with Runx2 (A, B), Twist (C, D), type I collagen (ColI; E, F), Osterix (Osx; G, H), Bsp (I, J) and Osteonectin (ON; K, L). Arrows indicate the oral (top) and aboral (bottom) sides of the osteogenic front of the mandible bone primordium. Yellow dashed lines (C, D) indicate the mandible bone.

of *Sox9* in the neural crest results in loss of Meckel's cartilage and malformation of mandible bone (Mori-Akiyama et al., 2003). As *Sox9* and *Ctgf* are not required for intramembranous ossification, the mandible defect in *Sox9* and *Ctgf* mutant mice strongly suggests that Meckel's cartilage has a critical role in mandible development, working as a guide for the formation of mandible primordium. In addition, loss of *Egfr* or *Shh* results in defects in Meckel's cartilage development and an under-developed lower jaw (Melnick et al., 2005; Miettinen et al., 1999). We conclude that growth factor signaling within CNC cells controls the development of Meckel's cartilage, which has a direct impact on the development of the mandible bone.

TGF-β-mediated Msx1 expression may control cell proliferation in the developing mandible

TGF-β IIR is specifically expressed during the condensation, proliferation and differentiation stages of mandible bone development, suggesting that it has a crucial role in regulating osteogenesis (Janssens et al., 2005). Our *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice have severe craniofacial bone defects in addition to that of the mandible. Previously, we have shown that TGF-β signaling is important for osteoprogenitor cell proliferation in frontal bone development (Sasaki et al., 2006). In the frontal bone primordium, FGF signaling induced by TGF-β is required for osteogenic progenitor cell proliferation. Based on this finding, we examined *Fgfr1* and *Fgfr2* expression in the mandible of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* samples by in situ hybridization, but found no difference from control samples. Previous study has shown that conditional inactivation of *Fgfr1* in the neural crest does not affect branchial arch development. Instead, FGFR1 controls the entry of CNC cells into the branchial arch non-cell-autonomously by creating a permissive environment (Trokovic et al., 2003). Taken together, TGF-β signaling within the CNC does not appear to control mandible development via FGFR signaling during craniofacial development.

Members of the *Msx* homeobox gene family are critical factors involved in craniofacial development. *Msx1* null mutant mice have a small mandible and missing alveolar bone (Satokata and Maas, 1994). Loss of *Msx1* results in a CNC cell proliferation defect (Han et al., 2003), similar to the cell proliferation defect in the mandible primordium of *Tgfbr2* mutant mice. *Msx2* null mutant mice do not have a mandible defect (Satokata et al., 2000). Recently, Ishii et al. reported that *Msx1/Msx2* double knockout mice have shortened mandibles, which are fused with the maxillary bone (Ishii et al., 2005). Apparently, *Msx1* and *Msx2* are expressed in pre-migratory and migratory neural crest cells and have major roles in regulating neural crest development (Chai and Maxson, 2006; Gajavelli et al., 2004; Takahashi et al., 2001). *Msx* genes are known for their essential roles in regulating the fate of CNC cells and osteogenesis during craniofacial development (Han et al., 2003). In this study, we found that *Msx1* was expressed in undifferentiated mesenchymal cells between Meckel's cartilage and the mandible bone at E13.5 and E14.5 in control mice, but was reduced in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice (Fig. 4). *Msx1* may function to induce osteoprogenitor cell proliferation under the control of TGF-β.

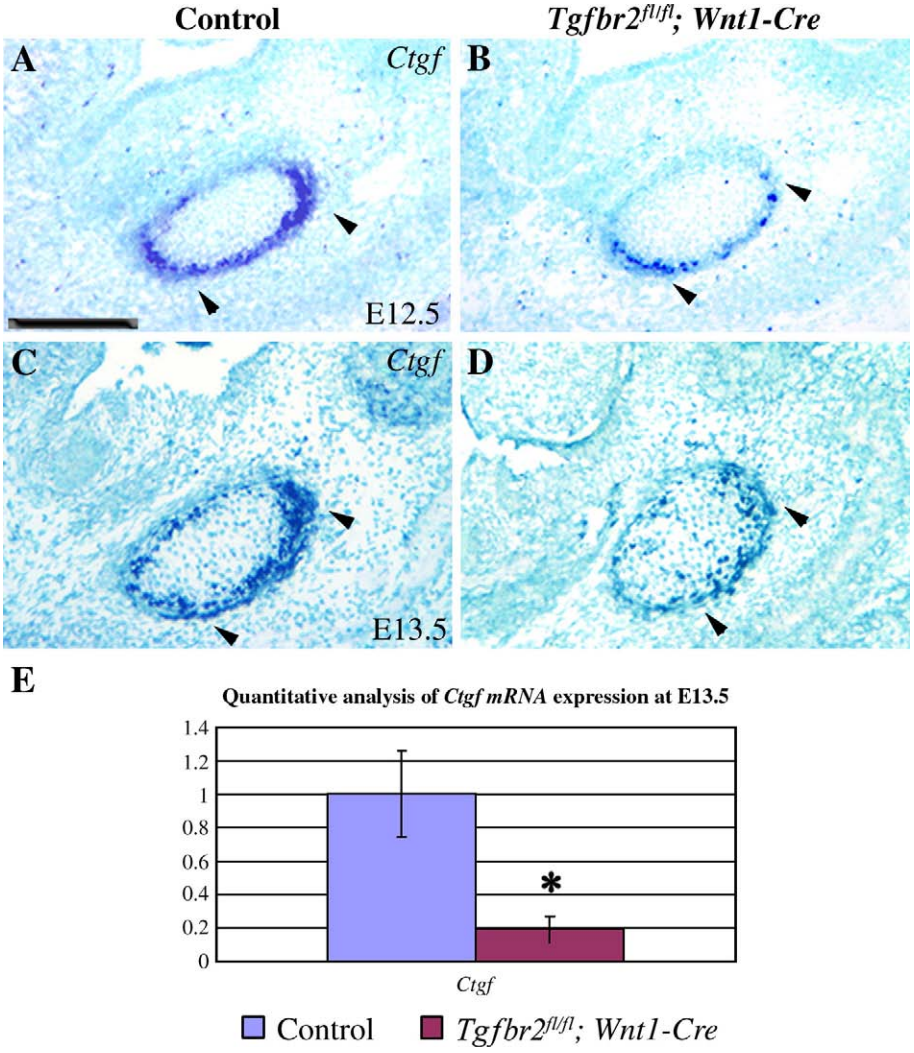


Fig. 6. Ctgf expression is down-regulated in the perichondrium of the Meckel's cartilage in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. (A–D) In situ hybridization of Ctgf in the perichondrium of Meckel's cartilage at E12.5 and E13.5 in control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. There is a significant reduction of Ctgf expression in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at E12.5 and E13.5 (arrowheads, A–D). (E) Quantitative analysis of Ctgf mRNA expression by real-time quantitative RT-PCR in Meckel's cartilage at E13.5. *Ctgf* expression is dramatically decreased in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples. Asterisks indicate statistical significance ($p<0.01$). Scale bar: 200 μm in panels A–D.

TGF-β-mediated CTGF expression controls cell proliferation during Meckel's cartilage development

TGF-β signaling plays an important role in the regulation of chondrocyte proliferation and differentiation (Serra et al., 1997). Previously, we have shown that TGF-β promotes chondrogenesis by selectively increasing the proliferation of CNC-derived chondrocytes in an in vitro mandibular organ culture model (Ito et al., 2002). In Meckel's cartilage of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice, cell proliferation activity was significantly reduced. Other studies have reported that *Col2a1-Cre*-mediated *Tgfbr2* conditional knockout mice do not appear to have any defect of mandible bone development (Baffi et al., 2004; Ovchinnikov et al., 2000). However, the *Col2a1-Cre* was not expressed in the perichondrium, so TGF-β IIR signaling remained intact there. The perichondrium surrounding the cartilage is thought to synthesize essential factors that regulate cell proliferation and differentiation in chondrogenesis (Lee et al., 2000; Liu et al., 2002; Mukherjee et al., 2005). Based on the differences in craniofacial defects between *Tgfbr2$^{fl/fl}$;Wnt1-Cre* and *Tgfbr2$^{fl/fl}$;Col2a-Cre* mice, we suggest that TGF-β signaling in the perichondrium plays a critical role in regulating the proliferation of chondrocytes through a possible paracrine mechanism. Thus, loss of TGF-β signaling in the perichondrium results in the under-development of the perichondrium and malformation of Meckel's cartilage.

CTGF is involved in a variety of developmental processes, including cell fate determination, cell migration, proliferation and differentiation (Chaqour and Goppelt-Struebe, 2006; Grotendorst, 1997). CTGF has been proposed as a downstream mediator of TGF-β action because its promoter contains TGF-β response elements (Leask et al., 2003). Loss of *Ctgf* results in multiple craniofacial defects, including a Meckel's cartilage malformation that is quite similar to that of the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant (Ivkovic et al., 2003). We have detected *Ctgf* expression in the perichondrium of Meckel's cartilage at E12.5

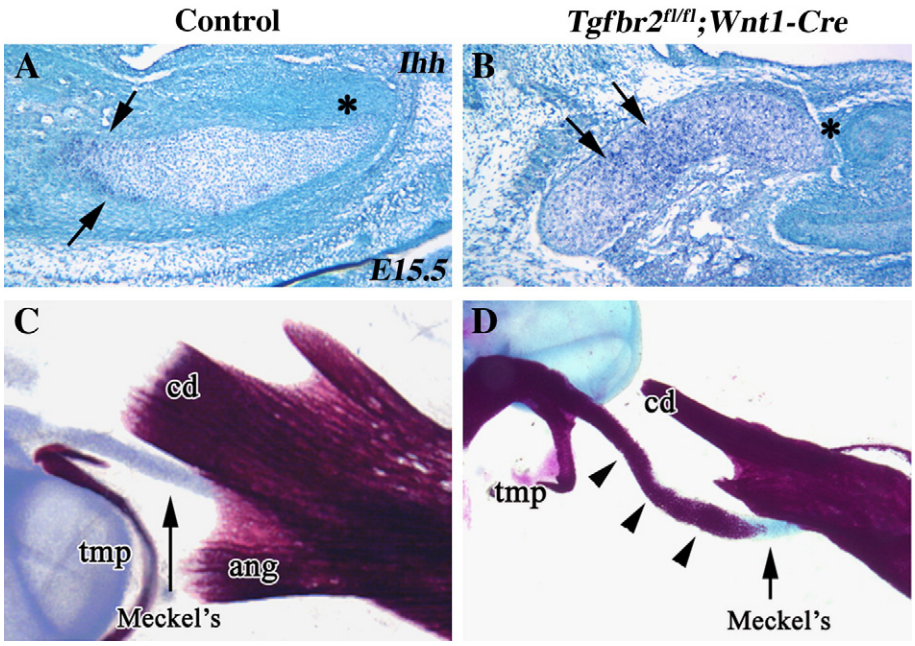


Fig. 7. Differentiation of chondrocytes in Meckel's cartilage is accelerated in *Tgfbr2$^{fl/fl}$;Wnt1-Cre*. (A, B) In situ hybridization of *Ihh* in Meckel's cartilage of control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at E15.5. *Ihh* is expressed in the middle region (arrow) but not in the distal region (*) of Meckel's cartilage in control mice. *Ihh* expression is expanded to the distal region of Meckel's cartilage in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. Asterisks indicate the distal end of Meckel's cartilage. (C) Skeletal staining of the proximal part of the mandible in the control sample. (D) Skeletal staining of the proximal part of the mandible in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. Abnormal ossification (arrowhead) is present around the proximal part of Meckel's cartilage (arrow). Scale bar: 200 μm in panels A–D. tmp, tympanic ring; cd, condylar process; Meckel's, Meckel's cartilage.

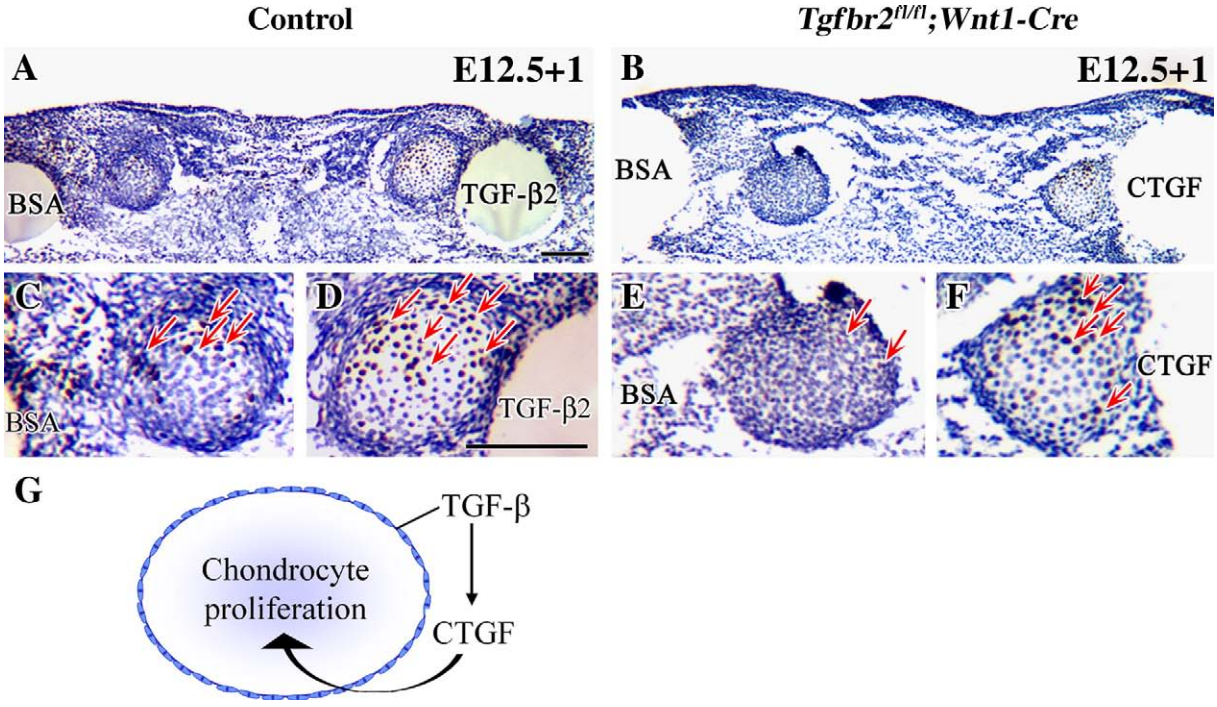


Fig. 8. CTGF is a downstream mediator of TGF-β signaling to control cell proliferation in Meckel's cartilage. BrdU labeling of cultured Meckel's cartilage from control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice at E12.5, treated with BSA, TGF-β2 or CFGF beads for 24 h (E12.5+1). (A, C, D) Control sample treated with BSA and TGF-β2 beads. The number of BrdU labeled cells (red arrows) is much greater in Meckel's cartilage treated with TGF-β2 beads. (B, E, F) *Tgfbr2$^{fl/fl}$;Wnt1-Cre* sample treated with BSA and CTGF beads. The *Tgfbr2$^{fl/fl}$;Wnt1-Cre* sample has few labeled cells with BSA beads but many more after treatment with CTGF beads (red arrows). (G) Schematic drawing demonstrates that TGF-β controls CTGF expression in the perichondrium to regulate chondrocyte proliferation during Meckel's cartilage development. Scale bar: 100 μm in panels A–F.

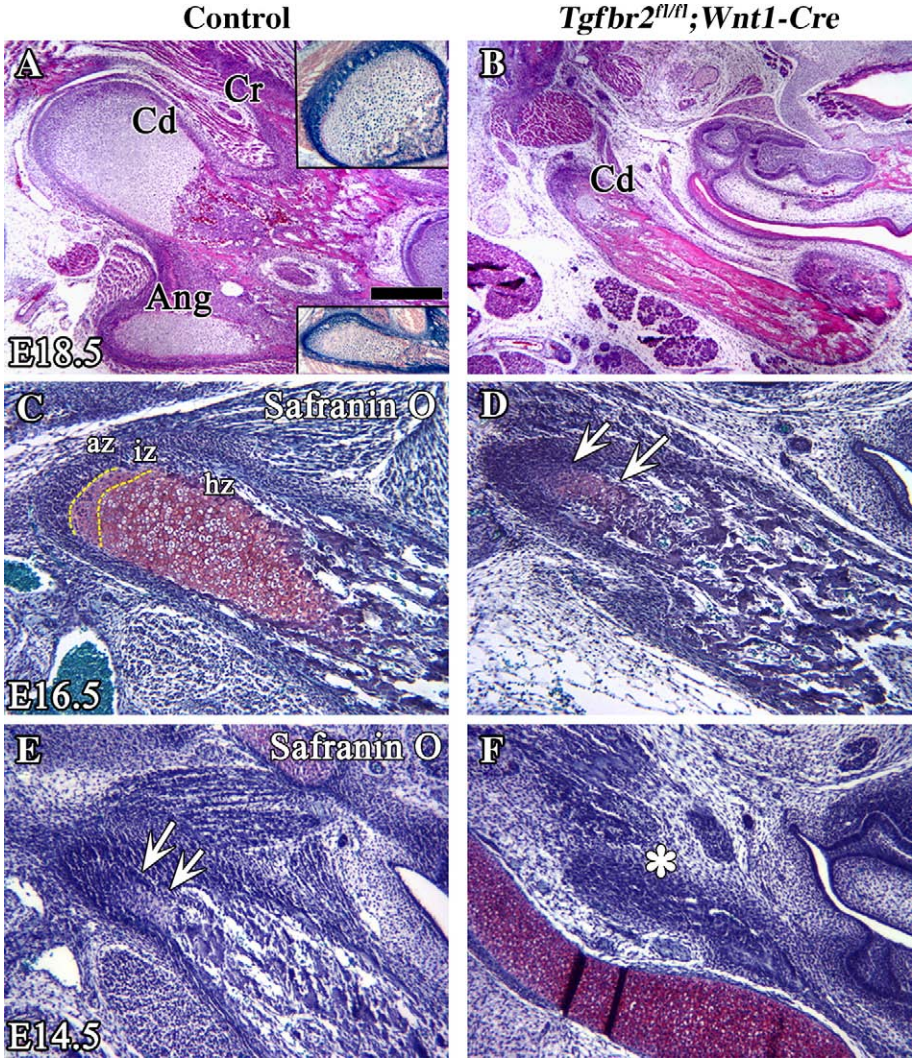


Fig. 9. TGF-β signaling is required for proper development of the condylar process. Histological analysis of the coronoid, condylar and angular processes in control and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice. (A, B) H&E staining shows clear zones of endochondral ossification in the condylar and angular processes in the control at E18.5. The insets show X-gal staining of the condyle and angular processes. The endochondral ossification of the condylar process is diminished in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant at E18.5. (C–F) Safranin-O staining of the condyle process at E16.5 (C, D) and at E14.5 (E, F). At E16.5, the three zones, articular, intermediate and hypertrophic, are visible in the control, but chondrogenesis of the condylar process is dramatically diminished and the hypertrophic zone is not detectable in the mutant (arrows). At E14.5, the condylar cartilage matrix is visible in control (arrows), but not in the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice (asterisk). Scale bar: 200 μm in panels A–H. Cd; condylar process, Cr; coronoid process, Ang; angular process, az; articular zone, iz; intermediate zone, hz; hypertrophic zone.

and E13.5, and in chondrocytes in Meckel's cartilage later. *Ctgf* expression in the perichondrium is significantly reduced in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice at E12.5 and E13.5. We also observed a reduction in proliferation activity in *Tgfbr2*$^{fl/fl}$*; Wnt1-Cre* mice at E12.5 and E13.5 when *Ctgf* is expressed specifically in the perichondrium. Finally, we also showed that exogenous CTGF can rescue the cell proliferation defect in Meckel's cartilage of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant samples. These findings suggest that CTGF signaling has a critical role in regulating proliferation of chondrocytes. Thus, we provide the first experimental evidence for a TGF-β-mediated CTGF signaling cascade that regulates craniofacial development.

In addition, we have also investigated the differentiation of chondrocytes in Meckel's cartilage. We found that *Ihh* expression, a marker for maturating chondrocytes (Chung et al., 2001; St-Jacques et al., 1999), was expanded in *Tgfbr2*$^{fl/fl}$*; Wnt1-Cre* mice. We conclude that the abnormal formation of Meckel's cartilage in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mice is the result of changes in both proliferation and differentiation.

TGF-β signaling and condylar development

During long bone endochondral ossification, TGF-β signaling plays an important role in the regulation of chondrocyte proliferation and hypertrophic differentiation (Serra et al., 1997). The mandibular condylar cartilage, which originates from the mesenchyme of the neural crest, serves as an important growth center in the developing mandible (Noden, 1975; Silbermann et al., 1987). The proximal part of the mandible is composed of the condyle, coronoid and angular processes, which develop through endochondral ossification, and these three processes are important for mandible function in conjunction with the

muscles of mastication. *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice have a defect in all three processes, with a gradient of increasing severity from the distal to the proximal region of the mandible (see Fig. 1E, F). Further study is needed to identify the source and functional mechanism of TGF-β signaling in regulating proximal region of mandible development. Nevertheless, mandible development appears to have a gradient requirement for TGF-β signaling, which functions as a morphogen in regulating mandible formation.

In this study, we focused on the condylar process defect in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mice. This defect started at a very early stage (before E14.5), and the zonation of the condylar process was completely lost at later stages. We found that TGF-β signaling during condylar process development is important for the differentiation of chondrocytes. We also detected changes in *Ihh* and *Pthrp* expression in Meckel's cartilage of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice. Interestingly, mutants of both of these genes have a defective condylar process (Shibata et al., 2000; St-Jacques et al., 1999 and our unpublished data). Therefore, we hypothesize that TGF-β signaling may control *Ihh* and *Pthrp* expression to regulate the development of the condylar process. Further investigation is necessary to explore this issue.

In summary, TGF-β IIR is specifically required for proliferation in both osteoprogenitor and chondroprogenitor cells. TGF-β-mediated CTGF signaling is crucial for the proliferation of CNC-derived chondrocytes in Meckel's cartilage. Exogenous CTGF can rescue the defect in cell proliferation in Meckel's cartilage. There are separate signaling pathways that rely on TGF-β signaling to control the proliferation of chondroblasts and osteoblasts during mandible development. Ablation of TGF-β signaling results in a failure of endochondral ossification in the condylar process. This animal model will provide useful information on the mechanism of TGF-β signaling in regulating mandible development.

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